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Remarkably low genetic diversity in the widespread cave spider *Phanetta subterranea* (Araneae, Linyphiidae)

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Abstract

Most cave-obligate species (troglobionts) have small ranges due to limited dispersal ability and the isolated nature of cave habitats. The troglobiontic linyphiid spider *Phanetta subterranea* (Emerton, 1875), the only member of its genus, is a notable exception to this pattern; it has been reported from more counties and caves than any other troglobiont in North America. As many troglobionts exhibit significant genetic differentiation between populations over even small geographic distances, it has been hypothesized that *Phanetta* may comprise multiple, genetically distinct lineages. To test this hypothesis, we examined genetic diversity in *Phanetta* across its range at the mitochondrial cytochrome c oxidase subunit I gene for 47 individuals from 40 caves, distributed across seven states and 37 counties. We found limited genetic differentiation across the species' range with haplotypes shared by individuals collected up to 600 km apart. Intraspecific nucleotide diversity was 0.006 +/- 0.005 (mean +/- SD), and the maximum genetic p-distance observed between any two individuals was 0.022. These values are within the typical range

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observed for other spider species. Thus, we found no evidence of cryptic genetic diversity in *Phanetta*. Our observation of low genetic diversity across such a broad distribution raises the question of how these troglobiontic spiders have managed to disperse so widely.

Keywords
Appalachians, genetic diversity, Interior Low Plateau, Linyphiidae, *Phanetta subterranea*

Introduction

Caves are populated by a diverse community of organisms, with more than 1,300 cave-obligate species (i.e., troglobionts) known from the United States alone (Niemiller et al. 2019). Because caves provide 'islands' of habitat for cave-limited species, and because troglobionts typically have limited ability to disperse through surface habitats, most troglobionts have small, restricted distributions, and many are restricted to a single or few geographically clustered cave systems. For example, 31% (218/710) of troglobionts in the Appalachians and Interior Low Plateau karst regions in the eastern United States are known from a single cave, with many other species limited to just a handful of nearby caves (Christman et al. 2016). Only a select few species have even moderately broad ranges, with just nine troglobionts (three arachnids, three hexapods, and three crustaceans) reported from more than 30 counties (Christman and Culver 2001).

Spiders are a significant component of cave biodiversity, with more than 100 troglobiontic spiders known from the United States (Niemiller et al. 2019) and ~1,000 troglobiont spiders described worldwide (Mammola et al. 2017). The best studied cave spiders in the eastern United States are from the genus *Nesticus*, which has diversified into three dozen cave and surface species across the southern Appalachians (Hedin and Milne 2023). As is often the case for troglobionts, cave-limited *Nesticus* species are characterized by small ranges (three species are known from just a single cave, and many others from just a handful of caves) (Hedin and Milne 2023). In cases where a *Nesticus* species is known from multiple caves, they often exhibit high genetic divergence between caves, even over short distances (Hedin 1997; Snowman et al. 2010; Balogh et al. 2020; Zigler and Milne 2022; Hedin and Milne 2023).

The linyphiid spider *Phanetta subterranea* (Emerton, 1875) (Fig. 1), the only member of its genus, is a small (1.5–2 mm in total length) troglobiont. They are found in multiple cave habitats, from near entrances to deep cave zones, and are often quite common (Poulson, 1977, 1981). They are thought to feed on springtails (Poulson, 1977, 1981). *Phanetta* exhibit variation in the degree of eye formation; most individuals have eyes, but in some cases eyes are nearly absent (Millidge, 1984). *Phanetta* can grow from hatching to full size in about four months and have a lifespan of about one year (Poulson 1981). Clutch size ranges from three to 16 eggs that are ~0.6 mm in diameter, and a single spider can lay multiple clutches within a year (Poulson 1975). Its range extends across two karst regions (the Interior Low Plateau and the Appalachians (Niemiller et al. 2019)) spanning a dozen states, and the species is known from more counties and caves

Figure 1. Subterranean Sheetweb Spider (*Phanetta subterranea*). Photo by Matthew L. Niemiller.

than any other troglobiont in North America (Christman and Culver 2001; Niemiller et al. 2013; Christman et al. 2016). Although widespread and common in caves of the eastern United States, *Phanetta* has never been reported from surface habitats.

Despite its remarkably broad range, nothing is known about genetic diversity in this species. It has been suggested that modern taxonomic study would result in the splitting of *Phanetta* into multiple species (Christman and Culver 2001). This scenario was observed in the Southern Cavefish (*Typhlichthys subterraneus*) species complex, which is known from the southern Interior Low Plateau, southern Appalachians, and Ozarks karst regions. Genetic analysis of *T. subterraneus* revealed nine genetically distinct lineages, including the identification of *T. eigenmanni* as a distinct species, and efforts to delineate and describe other lineages as distinct species are underway (Niemiller et al. 2012; Niemiller et al. 2013; Hart et al. 2023). Similar results have been reported for various troglobionts in other parts of the world (e.g., Lefébure et al. 2006; Zhang and Li 2014), including the cave beetle *Darlingtonea kentuckensis* from eastern Kentucky (Boyd et al. 2020).

In this study we investigated potential cryptic diversity in *Phanetta* across its broad distribution through genetic analysis of the mitochondrial cytochrome c oxidase subunit I gene (*COI*), a marker commonly employed in the study of genetic diversity in invertebrates. We sought to estimate genetic diversity and explore genetic structure within this spider while addressing the question of whether *Phanetta* represents a complex of morphologically similar but genetically distinct lineages, or a single genetic lineage connected through gene flow over broader spatial scales.

Methods

Geographic analysis

We surveyed the literature to compile a list of all known *Phanetta subterranea* occurrences. Resources consulted included Culver et al. (2000), Christman et al. (2016), and Zigler et al. (2020), as well as unpublished records from various cave biologists. We mapped the range of *Phanetta*, and our sampling sites (Fig. 2), using ArcGIS Online (https://www.arcgis.com/index.html). We calculated the range extent/extent of occurrence (EOO) for *Phanetta* using GeoCAT (https://geocat.iucnredlist.org/editor). Range extent/EOO is the area of a minimum convex polygon which contains all the sites of occurrence (Bachman et al. 2011).

Sampling

Phanetta were collected by hand between 1998–2023 from 40 caves in 37 counties across seven states (Alabama, Georgia, Illinois, Indiana, Kentucky, Tennessee, and Virginia) (Table 1) and two karst regions (the Interior Low Plateau and the Appalachians)

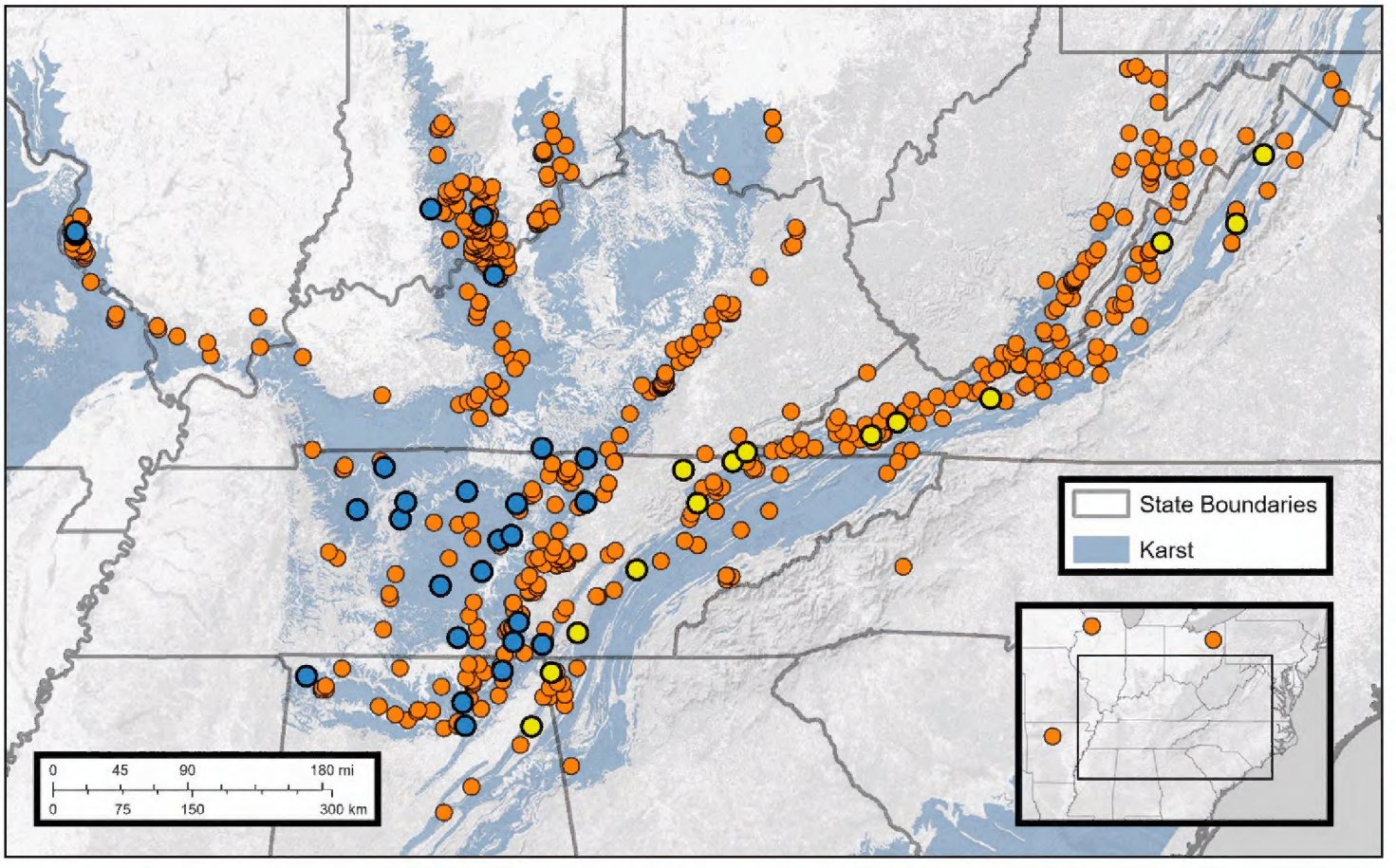

Figure 2. Range and sampling map. The distribution of *Phanetta subterranea* in the eastern United States. State boundaries are indicated by grey lines and karst terrain as blue-grey shading. Sites where *Phanetta* has been reported are indicated by orange points. Sites sampled in this study in the Interior Low Plateau karst region are indicated by blue points, and sites sampled in the Appalachians karst region are indicated by yellow points. This map includes ~600 georeferenced *Phanetta* sites. The inset indicates the extent of the main map, and includes three additional *Phanetta* sites (one in northeast Ohio, one in northwest Illinois, and one in central Arkansas), each more than 200 km from any other known *Phanetta* site, that are not visible on the main map.

(Fig. 2). We aimed to sample as broadly as possible, so generally limited our sampling to one cave per county. Specimens were preserved in 95% ethanol and stored at -20 °C until DNA extraction. Individuals were identified to species under the microscope; mature *Phanetta* females are easily identified by their distinctive epigynum (Emerton, 1875). In most cases, one spider per cave was sequenced; however, we sequenced two spiders from seven different caves. Collections were permitted by a variety of agencies (see Acknowledgements). Voucher specimens from this study are accessioned at the Auburn University Museum of Natural History.

Table 1. Sample sites for *Phanetta subterranea*.

State	County	Cave
Alabama	Colbert	Georgetown Cave
Alabama	DeKalb	Manitou Cave
Alabama	Jackson	Pseudo Lava Cave B
Alabama	Madison	Hering Cave
Alabama	Marshall	MacHardin Cave
Georgia	Dade	Howards Waterfall Cave
Illinois	Monroe	Danes Cave
Illinois	Monroe	Icebox Cave
Indiana	Dubois	Vowell Cave
Indiana	Harrison	Big Mouth Cave
Indiana	Washington	Twin Oaks Pit
Kentucky	Monroe	cave near Hestand, KY
Tennessee	Bedford	Fountain Cave
Tennessee	Campbell	New Mammoth Cave
Tennessee	Campbell	Norris Dam Cave
Tennessee	Cannon	Sycamore Creek
Tennessee	Claiborne	Obie Mill Cave
Tennessee	Coffee	Jernigan Cave
Tennessee	Davidson	Bull Run Cave
Tennessee	Davidson	Newsom Branch Cave
Tennessee	DeKalb	Indian Grave Point Cave
Tennessee	Dickson	Sinuous Stream Cave
Tennessee	Franklin	Tom Pack Cave
Tennessee	Grundy	Crystal Cave
Tennessee	Hamilton	Levi Cave
Tennessee	Lincoln	Kelso Saltpeter Cave
Tennessee	Marion	Pryor Cave Spring
Tennessee	Meigs	Sensabaugh Cave
Tennessee	Montgomery	Durham Cave
Tennessee	Overton	Mill Hollow Cave
Tennessee	Pickett	Frog Cave
Tennessee	Smith	New Salem Cave No. 1
Tennessee	Wilson	Spring Cave
Virginia	Bland	Repass Saltpeter Cave
Virginia	Highland	Five Springs Cave
Virginia	Lee	Grassy Springs Cave
Virginia	Rockingham	Massanutten Cave
Virginia	Russell	Bundys Cave No. 2
Virginia	Scott	Jesse Branch Cave
Virginia	Shenandoah	Flemmings Cave

Molecular techniques

We extracted DNA from specimens using the DNeasy Blood and Tissue Kit (Qiagen; Cat. No. 69504). We followed the manufacturer's protocol for extractions from whole or partial spiders. Polymerase chain reactions (PCRs) were prepared using the DNA extractions as template, GoTaq G2 Green Master Mix (Promega; Cat. No. M7822), dH_2O, and primers. Two different primer sets were employed to amplify a 651 base pair fragment of the mitochondrial *COI* locus. We initially used the primers HCO2198+M13F and LCO1490+M13R (modified from Folmer et al. (1994)), but we subsequently developed primers (PsHCO+M13F and PsLCO+M13R) that were more effective for amplifying *Phanetta* (Table 2). The PCR protocol was initial denaturation for 5 minutes at 95 °C, then 35 cycles of 15 seconds of denaturation at 95 °C, 30 seconds of primer annealing at 45 °C, and 60 seconds of extension at 72 °C. PCR products were visualized on 1% agarose gels. Successful PCRs were prepared for sequencing by treatment with Antarctic Phosphatase (New England Biolabs, Cat. No. M0289) and Exonuclease I (New England Biolabs, Cat. No. M0293). Samples were then sequenced on both strands using M13F and M13R primers on an Applied Biosystems 3730×l DNA Analyzer at the Keck DNA Sequencing Core of the Yale University School of Medicine (New Haven, CT).

Genetic analysis

We trimmed, assembled, edited, and aligned *COI* sequences using Geneious Prime (v. 2022.1.1). All sequences were submitted to GenBank (accession nos. PP815877–PP815923). We used MEGA11 (Tamura et al. 2021) to calculate genetic distances between sequences. P-distance, the genetic distance measure used here, is the proportion of nucleotides that differ between any two sequences. We used POPART (Leigh and Bryant 2015) to build a median joining tree (Bandelt et al. 1999) from the *COI* sequences. We looked for a pattern of isolation by distance by comparing linear geographic distance between sites and *COI* p-distance between individuals from those sites.

Results

Phanetta is known from 669 caves across 12 states and 155 counties (Fig. 2). When calculating the species range extent, we excluded three sites (one in northeast Ohio, one in northwest Illinois, and one in central Arkansas) because each was more than 200 km

Table 2. Primer names and sequences. Primers used to amplify a 651 bp fragment of the mitochondrial cytochrome oxidase I gene in *Phanetta subterranea*.

Primer name	Sequence (5'-3')	Reference
HCO2198+M13F	TGTAAAACGACGGCCAGTCGGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
LCO1490+M13R	CAGGAAACAGCTATGACCTAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
PsHCO+M13F	GTAAAACGACGGCCAGTACAAATCATAAAGATATTGGAAGTTTG	This study
PsLCO+M13R	CAGGAAACAGCTATGACCTTCAGGGTGACCAAAAAATCAAAATAA	This study

from any other known *Phanetta* site, raising the possibility of identification errors, or vagrancy. Even after excluding those sites, the species' EOO was 412,223 km^2 (Table 3).

We sequenced 47 *Phanetta* individuals from 40 caves across seven states and 37 counties (Fig. 2, Table 1). Full-length (651 bp) sequences were obtained from all individuals, and no indels or stop codons were observed. Genetic distances between *Phanetta* samples were low. Nucleotide diversity (π) in *Phanetta* was 0.006 $\pm$ 0.005 (mean $\pm$ SD), with a minimum pairwise p-distance of 0.000 and a maximum pairwise p-distance of 0.022 (Table 3). Twenty-one haplotypes were observed, and seven of these were shared, ranging in frequency from two to 14 individuals. The most common haplotype was present in *Phanetta* from Indiana, Illinois, Tennessee, and Virginia.

In seven cases, we sampled two individuals from the same cave. In six of those cases, the two individuals had identical *COI* sequences, and in the seventh case there was a single nucleotide difference between the two individual sequences. We found a positive correlation between the genetic distance between *Phanetta* individuals and the linear geographic distance between their sample sites (d.f. = 779, R^2 = 0.32, F = 373.7, significance F < 0.0001), indicating a pattern of isolation by distance, although the correlation was not particularly strong, and identical haplotypes were identified from sites as far as 600 km apart.

As one of the few troglobionts that is widespread across two major karst regions – the Appalachians and the Interior Low Plateau (Niemiller et al. 2019) (Fig. 2, Table 3) – *Phanetta* provided an opportunity to explore the effect of differing geologic history on genetic diversity within a single species. Haplotype diversity (h) was similar for the two karst regions ($h_{\text{Appalachians}}$ = 0.892, $h_{\text{Interior Low Plateau}}$ = 0.841) (Table 3). However, nucleotide diversity in *Phanetta* from the Appalachians ($\pi_{\text{Appalachians}}$ = 0.009 $\pm$ 0.006) was greater than in *Phanetta* from the Interior Low Plateau ($\pi_{\text{Interior Low Plateau}}$ = 0.003 $\pm$ 0.004) (Table 3). This pattern can be visualized in the haplotype network (Fig. 3) where haplotypes from the Interior Low Plateau are quite similar, mostly differing by just one or a handful of nucleotide differences. In contrast, haplotypes from the Appalachians typically differed from one another by multiple nucleotide differences (Fig. 3). Only one haplotype was shared by individuals from the Interior Low Plateau and the Ap-

Table 3. Distribution of and genetic diversity in *Phanetta* across karst regions. Range extent of *Phanetta* in the Interior Low Plateau and the Appalachians karst regions, and combined across the two regions, calculated as extent of occupancy (EOO). Measures of genetic diversity were calculated from all pairwise comparisons between individuals within the specified region. Based on cytochrome oxidase I sequences.

	Karst region		Combined
	Interior Low Plateau	**Appalachians**	
Range extent (EOO)	214,418 km^2	140,669 km^2	412,223 km^2
# of georeferenced sites	392	206	598
# of individuals sequenced	31	16	47
# of haplotypes	13	9	21
Haplotype diversity (h)	0.841	0.892	0.878
# of segregating sites	17	20	32
Nucleotide diversity (π) (+/- SD)	0.003 (+/- 0.004)	0.009 (+/- 0.006)	0.006 (+/- 0.005)
Maximum pairwise p-distance	0.015	0.020	0.022

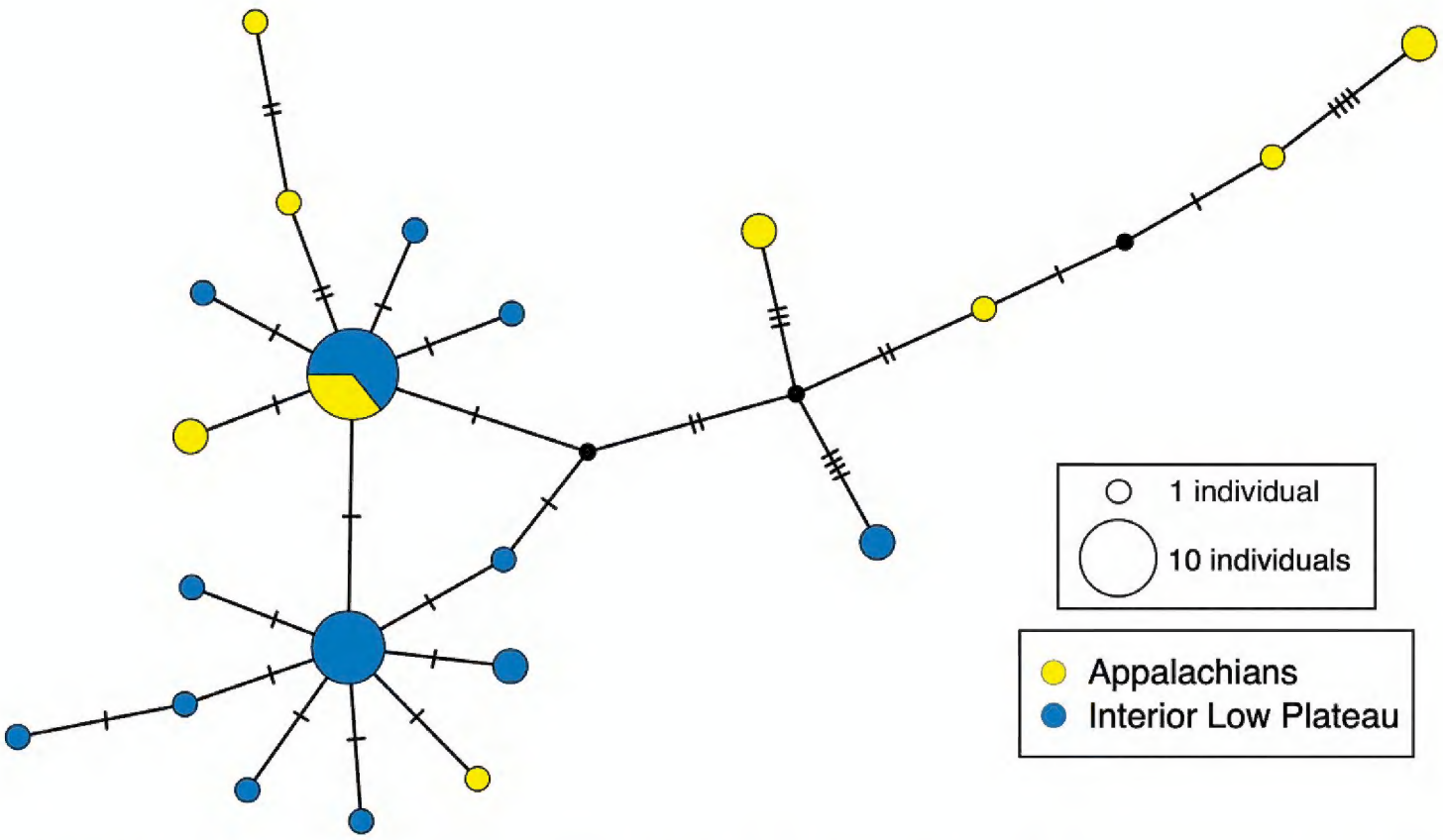

Figure 3. Median joining haplotype network for all *Phanetta* sequences. Haplotypes are indicated by circles and nucleotide differences between haplotypes are indicated by hash marks. Haplotypes are colored by karst region of origin as in Figure 2. Circle size indicates the number of individuals sharing a haplotype. The multicolored circle indicates the single haplotype shared by individuals from the Interior Low Plateau and individuals from the Appalachians.

palachians (Fig. 3). The higher genetic divergence observed in the Appalachians may be related to the great linear distance (~850 km) across which samples were collected (Fig. 2), although *Phanetta* does range across a greater area in the Interior Low Plateau (Table 3).

Overall, we observed remarkably low genetic variation across the broad range of *Phanetta*, with individuals from the Interior Low Plateau being particularly genetically uniform. *Phanetta* from the Appalachians exhibited slight genetic divergence from those from the Interior Low Plateau, and were also relatively more divergent from each other, but the overall genetic distance between any two *Phanetta* individuals was low. There was no evidence of cryptic genetic diversity within *Phanetta*.

Discussion

Phanetta subterranea is known from more caves and more counties than any other North American troglobiont. We aimed to determine whether *Phanetta* comprised a complex of genetically distinct lineages, or if it was genetically uniform across its range. After sampling 47 *Phanetta* individuals from 37 counties across seven states in the eastern United States, we found no evidence of cryptic genetic diversity. Genetic distances between sites were low, and haplotypes were shared across significant geographic

distances (up to 600 km). *Phanetta* from the Appalachians exhibited slight genetic differentiation from individuals from the Interior Low Plateau, as well as more genetic variation from each other (Fig. 3, Table 3). The higher nucleotide diversity observed in Appalachian *Phanetta* (Table 3) may be due to the highly faulted and fractured karst of the Appalachians causing greater isolation between *Phanetta* populations, whereas the lower nucleotide diversity observed in Interior Low Plateau *Phanetta* (Table 3) may reflect the more contiguous horizontal carbonate layers of this karst region, which could foster population connectivity.

We can compare our results to other spider species and to other troglobiont spiders from the eastern United States. A review of DNA barcoding efforts in spiders (Čandek and Kuntner 2015), using the same genetic marker (*COI*) that we employed in our study, provides a broad comparison. Summarizing results for 162 species, Čandek and Kuntner (2015) reported a mean intraspecific nucleotide diversity of 0.009, slightly higher than the 0.006 that we observed in *Phanetta.* Further, Domènech et al. (2022) used *COI* sequences to study genetic diversity in 371 spider species across a similarly-sized geographic region in Spain. They found a mean maximum intraspecific distance of 0.021, which is similar to the maximum intraspecific distance of 0.022 we observed for *Phanetta*. Clearly, the amount of genetic diversity we observed in *Phanetta* is not out of the ordinary range for a spider species.

In contrast, the *Phanetta* results are quite different from those observed in other troglobiont spiders for which genetic data are available. *Nesticus* spiders of the southern Appalachians exhibit high species diversity across a region smaller than the range extent of *Phanetta*, with many species having very small ranges (Hedin and Milne 2023). Multiple species of *Nesticus* are often found in close proximity, sometimes at sites just a few kilometers apart (Zigler and Milne 2022; Hedin and Milne 2023). Previous studies found considerable genetic diversity within species, even when those species ranges are very small. For example, Zigler and Milne (2022) reported *COI* genetic distances of 0.026 (in *N. cressleri*) and 0.031 (in *N. lula*) for cave populations less than 10 kilometers apart. As an additional example, *Nesticus barri* is known from around 60 caves on the southern Cumberland Plateau in Tennessee and Alabama. Genetic analysis of *N. barri* from a dozen caves found no haplotypes shared by individuals that were more than 12 km apart, and genetic distances (also for the *COI* locus) between individuals from different caves were as high as 0.045 (Snowman et al. 2010). These patterns strongly contrast with *Phanetta*, where haplotypes were shared by individuals as far as 600 km apart, and the maximum genetic distance (across a vastly larger geographic range) between individuals was 0.022.

Phanetta has never been reported from surface habitats, not even in a study of sinkholes within the range of the species (Lewis et al. 2020), and we have shown that populations across its range are genetically uniform. This raises the question as to how *Phanetta* has managed to colonize so many caves across such a broad area. We offer two, potentially complementary, hypotheses. First, as a tiny spider, it may be moving, undetected, through subterranean passageways such as caves and the interstitial spaces in shallow subterranean habitats (SSH) (Culver and Pipan 2019), including the

epikarst and the "milieu souterrain superficiel" (MSS), a layer of fractured rock beneath an insulating soil layer (reviewed in Mammola et al. 2016). In deeper cave habitats, troglobiont spiders have been shown to traverse through historical cave connections (Marsh et al. 2023). However, the distances traveled by historical *Phanetta* populations to form its current distributions are magnitudes larger than that studied by Marsh et al. (2023) and it is unknown if subterranean dispersal could fully explain its current range. Spiders have been collected within the MSS, especially in Europe (e.g., Růžička 1990, 1996; Růžička and Thaler 2002). However, studies on spiders from the MSS in North America are non-existent (Mammola et al. 2016).

A second possibility is that *Phanetta* disperses via ballooning, where spiders use their silken threads to be carried by the wind from one place to another (Greenstone et al. 1987). Studies of the diversity of ballooning spiders in the United States and Europe indicate that members of the family Linyphiidae are the spiders most commonly observed (Dean and Sterling 1985; Plagens 1986; Greenstone et al. 1987; Blandenier 2009; Blandenier et al. 2014), so it is not unreasonable to suggest *Phanetta*, a linyphiid spider, may also disperse in that way. If this is occurring, ballooning would probably have to be paired with at least some subsequent surface movement of individuals post-landing, as caves and cave entrances are relatively rare on the surface. Ballooning, which would allow the spiders to disperse across great distances, could explain the species' broad range, and the sharing of *COI* haplotypes between individuals collected as far as 600 km apart. However, the fact that *Phanetta* have never been observed on the surface weighs against the likelihood of ballooning as a method of dispersal.

This study could be extended in several ways. Further sampling of *Phanetta* from eastern Kentucky and from West Virginia would be valuable. We were unable to acquire samples from those areas. We also suggest searching for *Phanetta* from the three peripheral populations (Fig. 2) that we omitted from our estimation of range extent, as confirming or dismissing those observations would clarify the true range of the species. Two other linyphiid species – *Porrhomma cavernicola* and *Anthrobia monmouthia* – are wide-ranging troglobionts in eastern North America whose ranges overlap with *Phanetta* (Miller 2005a, 2005b). While neither is as common nor as wide-ranging as *Phanetta*, both are found across multiple states and karst regions, and genetic analyses of these species would provide an interesting comparison to the patterns we observed in *Phanetta*.

We also recommend exploring the possibility of ballooning in *Phanetta*. It might be possible to search directly for ballooning in *Phanetta* by setting aerial traps at the entrance of caves known to host *Phanetta*, aiming to catch any spiders leaving the cave by ballooning. Although some research on ballooning has been conducted in the United States (e.g., Dean and Sterling 1985; Plagens, 1986; Greenstone et al. 1987), none of these studies were done within the range of *Phanetta*. As a result, it remains unclear whether *Phanetta*, like many linyphiid species, disperses by ballooning. In addition, study of SSH within the range of *Phanetta* could clarify whether *Phanetta* are present in these habitats. In combination, studies of ballooning and SSH could support or reject our hypotheses for how *Phanetta* spread across such a large range.

In summary, we reject the suggestion that *Phanetta subterranea* contains cryptic genetic diversity and represents multiple species. Rather, it is a single, genetically uniform, species that has dispersed broadly across the caves of eastern North America. How it has managed to do this remains a mystery.

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